AD

GRANT NUMBER DAMD17-94-J-4258

TITLE: Function of Cell Cycle Control Proteins in Breast Cancer

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REPORT DATE: September 1997

TYPE OF REPORT: Annual

19980116 161

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release;
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DTIC QUALITY INSPECTED 3

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

1. AGENCY USE ONLY *(Leave blank)*

2. REPORT DATE
September 1997

3. REPORT TYPE AND DATES COVERED
Annual (1 Aug 96 - 31 Jul 97)

4. TITLE AND SUBTITLE
Function of Cell Cycle Control Proteins in Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4258

6. AUTHOR(S)
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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Harvard Medical School
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, MD 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Loss of the retinoblastoma tumor suppressor protein (pRb) can contribute to breast tumor formation. However, many breast tumors retain expression of normal pRb, indicating that other genetic events may interfere with the function of this growth regulatory protein. Overexpression of cyclin D1 is such an event, since high levels of this protein may lead to constitutive inactivation of pRb through phosphorylation. We have demonstrated that cyclin D1 in oncogenic in cultured cells, and are using this assay to define regions and functions of cyclin D1 needed for transformation. Interestingly, a previously defined pRb-interaction domain in cyclin D1 is dispensable for phosphorylation and transformation by cyclin D1, but apparently not in the closely related cyclin D2. Instead, loss of a cyclin D1 domain just C-terminal the pRb-interaction domain may inactivate the protein. Using our knowledge of cyclin D1 function from these mutants and others, we hope to eventually create animal models expressing cyclin D1 mutants that will allow a true in vivo determination of cyclin D1 functional domains. We have also produced and characterized a temperature sensitive pRb that can be used to identify gene targets of pRb. This mutant has revealed a cell-death effect resulting from pRb loss that may be due to the establishment of a pRb-directed, terminal cell cycle exit.

14. SUBJECT TERMS Breast Cancer, cyclin D1, retinoblastoma protein, transformation, tumor suppressor, oncogene

15. NUMBER OF PAGES
67

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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9/9/97

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INTRODUCTION

Breast cancer, like other cancers, results from the hyperactivity of growth-promoting oncoproteins and the loss of growth suppressing proteins (tumor suppressors). Many oncoproteins and several tumor suppressor proteins have been identified in recent years. Among the most commonly altered tumor suppressor proteins is the retinoblastoma protein, pRb. pRb function is lost in all retinoblastoma tumors, where it can lead to hereditary cancer, but is also involved in a variety of other tumors due to somatic inactivation. Reintroduction of the RB-1 cDNA into such cells inhibits their proliferation, supporting the role of pRb-inactivating mutations in formation of neoplastic cells (reviewed in Weinberg, 1991). This tumor-suppressive property of pRb is believed to result from pRb's ability to regulate progression through the cell cycle.

A central role for pRb in the control of cellular proliferation is also suggested by the observation that pRb is targeted by the oncoprotein products of several DNA tumor viruses. This interaction presumably serves to inactivate the growth-suppressive properties of pRb in infected or transformed cells. One mechanism by which oncoprotein-mediated inactivation may be achieved is through the dissociation of protein complexes between pRb and growth promoting molecules. For example, pRb has been reported to associate with the transcription factor E2F, which may be involved in the regulation of many genes required for DNA synthesis. The association of pRb with E2F may prevent the activation of these genes until the G1/S boundary, at which time the pRb/E2F complex dissociates, resulting in the release and activation of E2F. Because association of pRb with E2F seems to involve the same region of pRb (the "pocket") that is required for association with the viral oncoproteins, the binding of viral oncoproteins to pRb may release and activate E2F, resulting in the removal of a block to progression into S phase (reviewed in Tiemann, et al., 1997).

As is the case with a variety of human tumor types, some thirty percent of breast tumors show loss of pRb expression (Weinberg, 1991); however, other tumors have apparently wild-type pRb, and may have suffered alterations in one or another cellular proteins which interact with pRb. This may in turn lead to constitutive inactivation or circumvention of pRb function. A clue to the identity of such regulators of pRb is given by the fact that pRb is normally controlled by phosphorylation mediated by cyclin-dependent kinases (cdks; Lin, et al., 1991; Lees, et al., 1991). These cdks are controlled in turn by cyclins, regulatory subunits which lead to cyclic activity of their partner kinases. Work from many laboratories suggests that D-type cyclins in combination with cdk4 or cdk6 can initiate pRb phosphorylation in G1 and cyclin E/cdk2 complexes may continue or expand on this phosphorylation just prior to S phase entry (Ewen, et al., 1993; Kato, et al., 1993). Indeed it has been suggested that both cdk4 and cdk2 activity may collaborate to fully inactivate pRb prior to S phase entry (Hatakeyama, et al., 1994). The activity of these cyclin/cdk complexes is further regulated by positive and negative phosphorylation of the cdk subunit. In addition, several proteins have recently been identified that serve to stoichiometrically inhibit the function of cyclin/cdk complexes (reviewed in Morgan, 1995). Thus, these cyclin-dependent kinase inhibitors, or CKIs, together with cdk-modifying enzymes and cyclins

represent potential targets for oncogenic mutations that may lead to deregulated cell cycle progression.

Importantly, cyclin D1 has been shown to be overexpressed in some thirty percent of breast tumors (Lammie, et al., 1991; Schuuring, et al., 1992; Buckley, et al., 1993; Keyomarsi and Pardee, 1993), as well as in cancers of the parathyroid, blood and squamous epithelium (Motokura, et al., 1991; Rosenberg, et al., 1991a,b; Withers, et al., 1991). Thus, deregulated D-type cyclin expression may be oncogenic, leading to aberrant cellular proliferation perhaps by interfering with the function of pRb. We have shown that cyclin D1 can indeed act as an oncogene, cooperating to transform cultured cells in cooperation with a mutant adenovirus E1A oncoprotein which has lost the wild-type capacity to bind and inactivate pRb (Hinds, et al., 1994).

In a conceptually similar manner, loss of CKI function may also lead to loss of pRb function in pRb-positive tumors. p16INK4a, an inhibitor tailored specifically to prevent the function of cdk4 and cdk6, is deleted or mutated in many cancers, presumably leading to hyperactivity of the cyclin D/cdk4(6) complex that initiates pRb phosphorylation (reviewed in Weinberg, 1995). p16 is thought to act as a direct competitor of D-type cyclins for cdk4/6 association, preventing the activation of these kinases when conditions are inappropriate for cellular proliferation. Thus, it is clear that tumor cells exploit at least three mechanisms (Figure 1) to abrogate pRb function: elimination of pRb itself, elimination of the negative regulator p16, and overexpression of cyclin D1 (Weinberg, 1995).

We have recently shown that deregulation of additional members of the p16/cyclin D1/pRb pathway can also promote cellular proliferation in the face of growth suppressive influences. Using rat cells expressing a conditional allele of p53, we have found that overexpression of cdk4 or cdk6 can restore the ability to phosphorylate pRb and progress through the cell cycle. Cells overexpressing these kinase subunits can proliferate continuously in the presence of functional p53, in part because the p21 CKI is prevented from association with other cdk/cyclin complexes required for cell cycle progression (Latham, et al., 1996). However, because p53 has growth suppressive influences separate from p21 induction (Brugarolas, et al., 1995; Deng, et al., 1995), overexpressed cdk4 and cdk6 may have pleiotropic effects on these cells that circumvent unidentified functions of p53. In support of this is the observation that rat cells proliferating in the presence of overexpressed cdk4/6 contain complexes between cdk4/6 and p16 CKI family members that are perhaps contributing to the deregulated proliferation of these cells. Indeed, our preliminary experiments suggest that even nonfunctional cdk4/6 can promote pRb phosphorylation upon overexpression in human tumor cells, perhaps by competing with endogenous, functional cdk4/6 for inhibitory subunits.

Consistent with a role for cdk4 in human tumor formation, a mutant form of cdk4 that cannot bind p16INK4a has been found in melanoma cells (Wölfel, et al., 1995) . The molecular events outlined in Figure 1 may be oncogenic solely due to inactivation of pRb, and as such provide several alternatives to RB alteration in cancer. However, differences in the frequency of mutation of each pathway

member occur in distinct tumor types, suggesting that alterations in cyclin D/cdk4 activity may be more profound than loss of pRb in some cell types. Our current work is focused on understanding the mechanisms by which D-type cyclins and cdk4/6 can act as oncogenes.

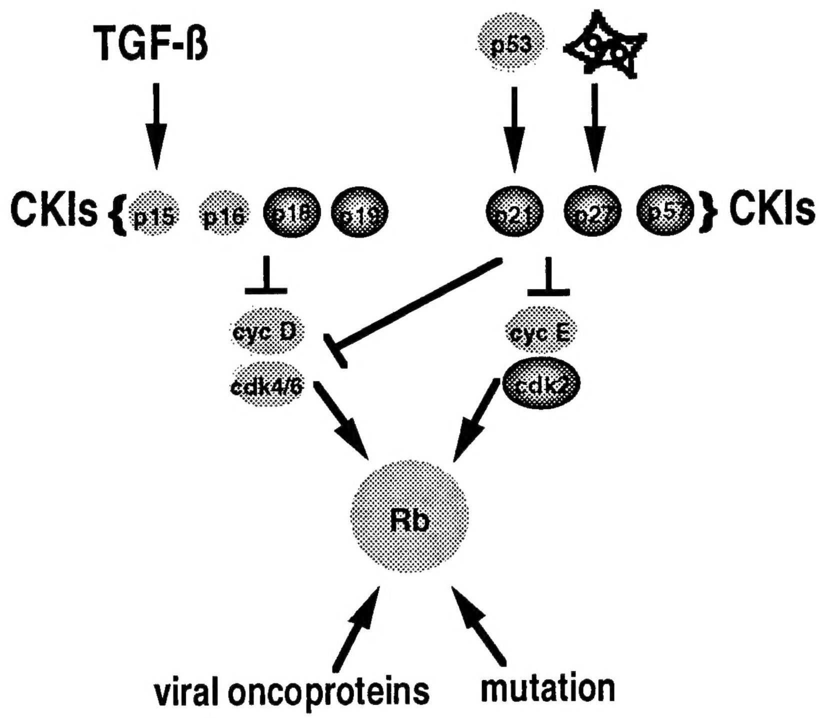

Figure 1. Interaction of cell cycle machinery, pRb and p53. Cyclin-dependent kinase (cdk) inhibitors (CKIs), cyclins and cdks involved in G1-S progression are shown. These proteins regulate the function of the Rb protein, a central target of oncogenic mutations. It has long been realized that such mutations inactivate pRb, and this can also be achieved by viral proteins, leading to uncontrolled proliferation. More recently, the cell cyclin components shown in gray have been demonstrated to be altered in tumors and/or function as oncogenes or suppressors in culture. Note that the CKIs are mediators of the growth suppressive influence of a variety of cell cycle inhibitors, such as TGF-ß, p53 and cell-cell contact. This pathway suggests that there are many ways to ultimately deregulate pRb, and most tumor cells may contain alterations in one of these components.

We and others have shown that D-type cyclins can physically associate with pRb in a manner analogous to the viral oncoproteins (Ewen, et al., 1993; Kato, et al., 1993; Dowdy, et al., 1993), a property of cyclin D1 that may explain its prevalence as a target of oncogenic activation. Paradoxically, however, this direct pRb binding is dispensable for transformation (Hinds, et al., 1994), and, as shown below, for phosphorylation of pRb. Intriguingly, we have recently found that a mutant cyclin D1 protein (KE) which is altered in the cdk-binding region not only

fails to transform primary cells, but is dominant over the wild-type protein in this capacity. Thus, cointroduction of mutant and wild-type cyclin D1 genes leads to no increase in transformation frequency, suggesting a dominant-negative function of the mutant protein. This mutant protein can be expressed in conjunction with wild-type E1A, however, and is thus not itself lethal to cells in the absence of overexpressed cyclin D1. More detailed understanding of the mechanisms behind the inhibition of cyclin function could lead to antiproliferative products using existing technologies and may be specific to those cells overexpressing certain cyclins, leaving normal proliferating cells relatively unaffected. The properties of D-type cyclins already uncovered in our prior research and those to be elucidated by the experiments described below provide an excellent opportunity for clinical intervention in aberrant cell cycle control, and could thus provide an important adjunct to the pharmaceutical treatment of human cancers. In addition, we seek to identify other upstream regulators or downstream targets of pRb which can inactivate pRb upon overexpression. Such candidate oncoproteins may be operative in the significant fraction of tumors which do not show direct inactivation of pRb. To address these issues, the following specific aims were originally proposed:

1) Use mutant pRb proteins that have been characterized for function as growth suppressors and substrates for the pRb kinase to determine if the association of D-type cyclins with pRb is required for pRb function or phosphorylation or both.

2) Identify a kinase activity precipitable with antibodies specific to a tagged D-type cyclin and use this functional assay to probe the effect of pRb binding on D-type cyclin function.

3) Introduce a dominant-negative D-type cyclin into cells transformed by wild-type D-type cyclins and control cells in effort to specifically prevent the proliferation of the transformed cells.

4) Identify other substances, e.g. antisense oligonucleotides or interfering peptides, which could cause cessation of proliferation of cyclin D-transformed cells in vitro and in vivo.

5) Use cell lines temperature-sensitive for growth due to the expression of temperature-sensitive pRb to identify upstream inactivators and downstream targets of pRb.

RESULTS AND FUTURE DIRECTIONS

Specific aims 1 and 2: Mapping of pRb and cyclin D1 domains required for transformation.

Because catalytic partners of cyclin D1 were identified as cdk4 and cdk6 several years ago, it was unnecessary to develop the assay proposed in aim 2.

Further, it was shown that the absence of pRb or its functional disruption by viral oncoproteins leads to a loss of cyclin D/cdk complexes, although the mechanism of this remains unclear (Bates, et al., 1994). We have therefore de-emphasized aim 2 and focus our efforts on cyclin D1 function in transformation on aim 1. In this case, despite a clear role for mediating cyclin D/pRb interaction in vitro (Dowdy, et al., 1993; Ewen, et al., 1993; Kato, et al., 1993), it is unclear what the role for the LXCXE domain is in vivo, since its disruption by point mutation often does not lead to functional inactivation (Dowdy, et al., 1993; Hinds, et al., 1994).

Thus, although it is clear that cyclin D1 can function as an oncogene and does so at least in part by interfering with the pRb protein, the exact mechanism by which this is achieved is unclear. For example, cyclin D1 may simply activate its cognate kinase partners, cdk4 and cdk6, to directly phosphorylate pRb. Alternatively, excess cyclin D1/cdk complexes may compete with or lead to inactivation of any of a variety of CKIs now known to regulate cdk activity. Finally, although the absence of pRb seems to negate the need for cyclin D1 in tumor cells (Lukas, et al., 1995; Parry, et al., 1995), cyclin D1/cdk complexes may have other cellular targets. Examples of such targets are the pRb homologues p107 and p130 (Beijersbergen, et al., 1995). These proteins are homologous to pRb in the region used to associate with viral oncoproteins. Indeed, these proteins were first identified due to their association with E1A. It is now clear that p107 and p130 associate with transcription factors capable of binding to the E2F DNA site (Shirodkar, et al., 1992; Devoto, et al., 1992; Cao, et al., 1992; Cobrinik, et al., 1993), thus the homology to pRb is functional as well as physical. However, the E2F proteins that associate with pRb do not appear to interact with p107 or p130 in cells. It is therefore likely that pRb, p107 and p130 are members of a family of proteins which regulate the function of a family of E2F transcription factors whose roles may or may not overlap in cell cycle control. Deregulated phosphorylation of these targets and pRb together may be more profoundly oncogenic than deletion of pRb alone, consistent with the high incidence of cyclin D1 overexpression in certain tumor subsets, such as is the case in breast cancer. We are attempting to identify the functional regions of cyclin D1 operative in transformation and correlate these with an induction of pRb phosphorylation.

Work in progress outlined in last year's progress report has demonstrated that complete elimination of the LXCXE domain in both cyclin D1 and cyclin D2 has no effect on the ability of these cyclins to activate cdk4 or cdk6 "in vitro", that is in cotransfection assays. Thus, whether pRb is supplied to cells in transfections or used as a synthetic substrate in in vitro kinase assays, complexes containing D-cyclins lacking the LXCXE domain can efficiently phosphorylate pRb. Interestingly, the ability of these mutant cyclins to bind to pRb has been less clear. In several experiments, LXCXE deletion mutants appeared to associate with pRb as efficiently as wild-type cyclin D proteins, although point mutations in the domain eliminated binding, as has been reported (Dowdy, et al., 1993; Ewen, et al., 1993). However, it has proved difficult to repeat these experiments cleanly due to nonspecific binding of D cyclins to mutant pRbs or GST proteins, despite careful experimental procedure. In addition, the interaction of these proteins in cell lysates, for example after transfection or in stable cell lines, is only rarely observed We believe that "stable" complexes described previously may only be

achieved in vitro or under highly specific conditions, and are therefore no longer confident in the information to be gained from mapping interaction domains until we better understand the nature of the interaction. Therefore, we have elected to focus on the biological properties of the mutant cyclin D proteins in functional assays as described below.

Mutant cyclin D1 proteins lacking the LXCXE domain, the ability to activate cdk4/6, or N-terminal regions between the LXCXE domain and the cyclin box are being tested in transformation and growth arrest assays. As was discussed in the previous report, and consistent with earlier results using the point-mutant protein, cyclin D1 lacking LXCXE was found to be fully functional in transformation. In contrast, initial experiments suggested that cyclin D2 lacking LXCXE is poor at transformation, indicating possible functional differences between the two cyclins. These experiments are continuing; however, at this point it is clear that the LXCXE domain of cyclin D1 is dispensable for kinase activation, phosphorylation of pRb in vitro and transformation of primary cells.

We have employed these cyclin proteins in a second growth assay with interesting results. This assay tests the ability of the cyclin protein to disrupt growth arrest caused by the transient expression of pRb in SAOS-2 human osteosarcoma cells. These cells undergo a cell cycle arrest in G1 and a distinct morphological alteration upon reexpression of pRb (Hinds, et al., 1992). Because the morphologically altered cells ("flat cells") can be counted, this assay allows a quantitative, visual assay for the ability of a co-introduced expression construct to disrupt pRb function. Previously, it had been found that cyclins A and E could disrupt flat cell formation with concomitant induction of pRb phosphorylation. Cyclin D1 also reduced flat cell formation, but no evidence of pRb hyperphosphorylation was seen (Hinds, et al., 1992). We have begun to use this assay for D-type cyclin mutants in effort to identify the function(s) of D-cyclins that disrupt pRb function in SAOS-2 cells.

Results from SAOS-2 cotransfections show that pRb-induced flat cells can be efficiently disrupted by LXCXE point mutant cyclin D1 as previously reported (Dowdy, et al., 1993), and further, the complete disruption of the LXCXE domain has no effect on the ability of cyclin D1 to prevent growth arrest. Thus, as with transformation and phosphorylation, the LXCXE domain is dispensable in functional assays of cyclin D1. Interestingly, we find that cyclin D2 is very poor at preventing flat cell formation in this assay. Further, the weak reduction in flat cells seen with wild-type cyclin D2 is completely lost by either point mutation or deletion of the LXCXE domain. Cyclin D1 may thus have a unique effect in this assay, independent of the LXCXE domain, and likely independent of pRb phosphorylation since we do not observe phosphorylation of pRb in these assays. A further interesting point arising from these assays is that a nonfunctional cyclin D1, called KE, that cannot activate cdk4/6, also fails to reduce flat cell formation caused by pRb. Thus, while there is no indication that pRb is phosphorylated by the cointroduction of cyclin D1 alone, kinase activation by cyclin D1 appears to be required for the interruption of pRb function in this assay. This raises the possibility that kinase targets other than pRb can be affected in these

experiments and that their phosphorylation may lead to an insensitivity to at least some aspects of pRb function. This possibility is being actively pursued.

The experiments described above will result in a clear picture of the role of the LXCXE domain in antagonizing pRb function in several in vitro (cell culture) assays. In general, they suggest that this domain is dispensable for in vitro pRb kinase activity and for cyclin D1 function in proliferation. However, a drawback of these experiments is that they all result from significant overexpression of D cyclins. This level of expression may override a need for the LXCXE domain that would be seen at endogenous levels of expression. We feel that the best way to examine this issue is to express cyclin D1 mutants under endogenous control mechanisms. To do this, we have designed a targeting vector that will allow integration of wild-type or mutant cyclin D1 cDNA in the mouse genomic cyclin D1 locus, disrupting this gene and allowing expression of the product of the cDNA. Generation of mice expressing cyclin D1 only from these engineered alleles will allow us to examine the ability of mutant forms of cyclin D1 to "rescue" the phenotypes caused by disruption of the mouse cyclin D1 gene (Sicinski, et al., 1995). This work will be done in collaboration with Piotr Sicinski, who has recently joined the Harvard Medical School Pathology faculty at the Dana-Farber Cancer Institute. We anticipate that this carefully controlled expression of mutant cyclin D1 will reveal any role of the LXCXE domain in breast development, which is profoundly defective in cyclin D1 knockout mice, and will allow the generation of cultured cells expressing the mutant protein at normal levels. These cells will allow careful comparisons of the biochemical properties of mutant cyclin D1 and better reveal the role of the LXCXE domain.

Specific aims 3 and 4: Use of dominant-negative cyclin D1 and molecular mimics thereof.

Our previous and ongoing work has shown that the cyclin D1 mutant KE is not only inactive for kinase activation and transformation, but can act as a dominant-negative in transformation assays (Hinds, et al., 1994 and unpublished results). As part of the studies described above and in the previous report, we have found that KE can bind to cdk4 with wild-type efficiency in transfections into human tumor cells, but the resultant complex is catalytically inactive. In contrast, in E1A-transformed BRK cells, KE appears associate with kinases only weakly in comparison to the wild-type protein. Finally, work in collaboration with Dr. Steve Dowdy suggests that KE can make complexes with cdks in most cells tested; it is possible that the expression of E1A in BRK cells compromises complex formation between D cyclins and cdks and accentuates a minor difference in affinity that is usually of little consequence.

We have continued to investigate the utility of dominant-negative cyclin D1 in human tumor cells. First, we have attempted to dissect the mechanism of dominant-negativity by co-introducing KE and wild-type cyclin D1 in SAOS-2 cells along with cdk4 to demonstrate KE-induced inactivation of cdk4. Surprisingly, we found that KE could not act as a dominant negative in this assay. We surmised that excess cdk4 resulting from transfection might prevent sequestration of this kinase subunit by KE, so attempted to perform similar experiments in U2OS cells,

which express wild-type cyclin D1 and functional pRb. Here again, however, we have seen little effect of KE on U2OS cell proliferation, suggesting that cdk4 and cdk6 function is not greatly affected by KE expression. Biochemical analyses of the complexes formed in these experiments has not yet been performed, so the ability of KE to compete for cdk4 in this assay cannot be determined. Clearly, more work is required to define the conditions and properties of KE that allow it to act in a dominant fashion before the goals of these aims are achieved. We plan to continue these studies by comparing kinase activities in co-transfected tumor cells that seem resistant to KE and in primary BRK cells, where the dominant-negative effect was first seen to determine if dominant kinase inactivation is cell-type specific. In addition, the KE mutation has been introduced into cyclin E, the activity of which is needed in all cells and which will allow a test of our mechanistic preconceptions about the function of this mutation.

Specific aim 5: Use cell lines temperature-sensitive for growth due to the expression of temperature-sensitive pRb to identify upstream inactivators and downstream targets of pRb.

Considerable progress has been made in the characterization and utilization of the temperature-sensitive allele of pRb (tspRb) called XX668. Briefly, this allele encodes a pRb that can very poorly induce flat cells, G1 arrest or colony reduction at 37° C, but does so with wild-type efficiency at 32.5°C. Further, the protein cannot repress E2F-dependent promoters at 37° C but is functional for this property at 32.5°C. Most interestingly, when tspRb is inactivated by temperature upshift to the nonpermissive temperature, BrdU incorporation is seen within 24 hours, indicating that the pRb-induced block to S phase is reversible. However, these cells do not go on to proliferate as do their untransfected counterparts but rather die shortly after S phase entry. This death is apoptotic as determined by FACS analysis and the ability of bcl-2 and E1b19K to overcome the death phenotype. Thus, establishment and reversal of pRb function in SAOS-2 osteosarcoma cells does not restore the original tumorous phenotype of these cells, but results in cell death. We believe that some program or downstream function of pRb manifested after long-term (several days) expression of pRb in growth arrested cells leads to a continuous anti-proliferative signal that conflicts with the re-establishment of S phase signals upon pRb inactivation. A manuscript detailing this work can be found in the Appendix and has been submitted to EMBO Journal.

We are using this tspRb in several ways. First, we are further investigating the defect in tspRb at 37°C that disallows E2F-dependent transcriptional repression. We have found that tspRb interacts with E2F equally at both temperatures, and so an inability to bind E2F is apparently not at the heart of tspRb's inaction at 37°C. Instead, it is possible that the transcriptional repression function of the pRb pocket is specifically defective. To test this, we are constructing GAL4-DNA binding domain constructs that contain the pRb and tspRb pocket to test for E2F-dependent transcriptional repression. It is possible that an unidentified cellular cofactor must bind to the pRb pocket to allow repression, and this binding may be temperature sensitive in tspRb. We

anticipate that the use of tspRb in these types of experiments will be useful in dissecting the function of the pRb pocket as a transcriptional repressor.

A second use of tspRb relies on cell lines that have been established in the SAOS-2 background. Several of these undergo growth arrest upon induction of pRb function, but only some undergo the morphological alteration characteristic of transiently transfected cells. The different phenotypes of these stable cell lines will allow us to not only examine the cell death resulting from pRb re-inactivation, but will allow us to identify genes that are turned on and off as pRb is activated and inactivated. We may thus be able to identify genes involved in proliferation that are directly controlled by pRb, but further may also identify genes involved in the phenotypic change induced by pRb. This can be achieved by comparing expression profiles in cell lines with and without morphological alteration. We anticipate initially using standard subtractive hybridization techniques to achieve these goals, but will consider the SAGE technique if cost allows.

Finally, we wish to introduce tspRb into other cell types in attempt to identify cell type specific changes caused by pRb. For example, the flat cell phenotype caused by pRb in SAOS-2 cells may be the result of some kind of senescence, but could also reflect an attempt of the cells to differentiate once pRb function has been restored. Such effects in other cell types could make a differentiative role for pRb more obvious. We plan to introduce tspRb into MDA-MB-468 breast tumor cells which lack functional pRb. A functional analysis of such transfectants could be quite useful in elucidating a role for pRb in terminal differentiation of breast epithelial cells.

CONCLUSIONS

Our ongoing work characterizing the method of action of cyclin D1 as an oncogene will be useful in identifying the functions of cyclin D1 that may serve as targets for anti-tumor approaches. Preliminarily, it seems that cyclin D1 differs from cyclin D2 in its biochemical effects on pRb in overexpression systems. These differences may explain the common observation of cyclin D1 as a human oncogene, in contrast to the rare incidence of cyclin D2 overexpression. Such differences are what we hope to exploit to reverse the aberrant proliferation resulting from cyclin D1 overexpression. The expression of cyclin D1 mutants in animal model systems seems necessary to achieve a clear understanding of the full role of cyclin D1 in proliferation. The ability to study this in breast development and other phenotypic consequences is exciting and made possible by a collaboration with Piotr Sicinski.

TspRb shows great promise as a reagent to unravel the specific effects of pRb on terminal cell cycle exit. Our present results with this reagent suggests that tspRb can cause a readily reversible block to S phase entry, but also causes more durable changes in the cell that result in death once pRb is inactivated. Such properties, if also seen in other cell types, could be quite useful in identifying gene targets and properties of pRb that influence terminal differentiation.

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APPENDIX: F. Tiemann and P.W. Hinds. Induction of DNA synthesis and apoptosis by regulated inactivation of a temperature-sensitive retinoblastoma protein. EMBO J, Submitted.

Induction of DNA synthesis and apoptosis by regulated inactivation of a temperature-sensitive retinoblastoma protein.

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Running Title: temperature-sensitive pRb mutant

Key Words: pRb; temperature-sensitive mutant; apoptosis; growth suppression; E2F-dependent gene repression

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Abstract

The retinoblastoma protein, pRb, controls entry into the S phase of the cell cycle and acts as a tumor suppressor in many tissues. Re-introduction of pRb into tumor cells lacking this protein results in growth arrest, due in part to transcriptional repression of genes required for S phase. Several studies suggest that pRb may also be involved in terminal cell cycle exit as a result of the instigation of senescence or differentiation programs. To better understand these multiple growth inhibitory properties of pRb, a temperature-sensitive mutant of pRb has been produced. This tspRb induces G1 arrest and morphological changes efficiently at the permissive temperature of 32.5˚C, but is weakly functional at 37˚C. Consistent with this, tspRb is compromised in nuclear association and E2F regulation at the nonpermissive temperature, but regains these properties at 32.5˚C. Serial activation and inactivation of tspRb in SAOS-2 cells does not allow proliferation, but rather leads to apoptotic cell death. Transient activation of pRb may kill tumor cells by establishing a conflict between persistent proliferation-inhibitory signals and renewed deregulation of pRb targets such as E2F, and may thus be a more potent means of eliminating these cells than through simple re-introduction of the tumor suppressor gene.

Introduction

Deregulation of cell cycle control resulting in inappropriate cell proliferation is one of the hallmarks of cancer. Tumor cells frequently have acquired damage to checkpoint genes that regulate progression through the cell cycle. Oncogenic processes thereby target regulators of G1 phase progression and the G1-to-S-phase transition. During G1 phase, cells respond to extracellular signals by either advancing through or withdrawing from the cycle into a resting state (Sherr, 1996). Self-renewing mammalian cells make this decision about growth versus quiescence as they pass the so-called restriction point (R) late in G1 (Pardee, 1989; Weinberg, 1995).

The retinoblastoma susceptibility protein, pRb, functions in a pathway that regulates passage of cells through the restriction point and into S phase. pRb exerts its function in part by controlling a family of heterodimeric transcription factors, collectively referred to as E2F, that can transactivate genes important for the G1 to S phase transition. Hypophosphorylated pRb binds to E2F, resulting in a transcriptional repressor complex that constrains expression of E2F target genes. Phosphorylation of pRb in mid to late G1 releases E2F from the complex, which now can be transcriptionally active (Weinberg, 1995; Sherr, 1996). Targeted deletion of the E2F-1 gene suggests that E2F-1 is necessary for the proliferation of some cells, but actually acts as a tumor suppressor in other cell types. This dual role for E2F-1 may result from an ability of E2F-1/pRb complexes to repress genes involved in apoptosis as well as genes whose products are needed for DNA synthesis (Qin et al., 1994; Field et al., 1996; Yamasaki et al., 1996; Hsieh et al., 1997; Phillips et al., 1997).

Phosphorylation of pRb and concomitant relief of E2F repression is catalyzed by cyclin D dependent kinases (cdk4 and 6) in response to extracellular signals. The activity of these kinases is in turn inhibited by proteins of the INK4 family (Sherr, 1996). Disruption of this so-called "pRb pathway" is a frequent event during the pathogenesis of a variety of human tumors (Tiemann et al., 1997), highlighting the importance of the integrity of this pathway to the prevention of tumor formation, as well as pRb's central role in it.

The function of pRb is lost in all retinoblastomas, where it can lead to hereditary cancer, but is also involved in a variety of other tumors, including small cell lung carcinomas, breast carcinomas, osteosarcomas, bladder carcinomas, prostate carcinomas and cervical carcinomas (Dyson et al., 1989; Horowitz et al., 1990; zur Hausen, 1991). Several different types of inactivating mutations in the *RB1* gene have been described, almost all of which specifically alter or eliminate the "pocket" domain of pRb. The pocket domain was first functionally characterized as the minimal region of pRb that is necessary for binding to the viral oncoproteins E1A, E7 and large T antigen. Thus, both oncogenic mutations and viral oncoprotein binding target the same subdomain of pRb, strongly suggesting a critical role for this domain in tumor suppression (Tiemann et al., 1997). Indeed, the pocket domain is sufficient for the repression of gene expression, but an extra C-terminal stretch of the pRb protein is necessary for E2F binding and growth suppression (Qin et al., 1992; Hiebert, 1993). pRb shares the pocket domain with two other related proteins, p107 and p130, which also bind and regulate E2F activity, but which have not yet been shown to be mutated in cancer (Ewen et al., 1992; Cobrinik et al., 1993; Hannon et al., 1993; Li et al., 1993; Mayol et al., 1993). Although the pRb/E2F paradigm leads to a simple model for growth regulation through the inactivation of E2F-responsive genes, the identity of genes

actually regulated by pRb is far from fully elucidated. The presence of p107 and p130 together with pRb in all cells complicates any simple model of pocket protein-E2F function, since these molecules may differentially regulate subsets of E2F-site containing genes (Hurford et al., 1997). Further, pRb interacts with many proteins besides E2F, many of which can themselves directly influence gene expression (reviewed in Taya, 1997). Among these are differentiation-specific transcription factors that are activated by pRb, rather than repressed, emphasizing a potential role for pRb in the process of terminal differentiation. Additionally, there is evidence that pRb may inhibit apoptosis (Haas-Kogan et al., 1995; Berry et al., 1996; Fan et al., 1996; Macleod et al., 1996; Shan et al., 1996; Hsieh et al., 1997) and somehow regulate replicative senescence, a phenomenon antithetical to tumor cell formation (Smith and Pereira-Smith, 1996).

In vivo evidence for a role of pRb in terminal cell cycle exit comes from mouse models in which both RB1 alleles have been replaced with defective alleles by means of homologous recombination techniques. Mice homozygous for defective RB1 die in utero at about the fourteenth day of gestation from abnormalities in the blood and the liver accompanied by extensive cell death in the central nervous system (Clarke et al., 1992; Jacks et al., 1992; Lee et al., 1992). Although pRb is also absent in all other tissues of the mouse embryo, most tissues develop grossly normally. Nevertheless, loss of pRb may lead to more subtle alterations in differentiated cell properties that have unknown consequences because of the inability to study them in dead embryos. For example, although muscle development appears normal in RB1 nullizygotes, myotubes produced from such embryos fail to enter a truly "terminal" state, since their nuclei can be induced to enter S phase, unlike those from genetically normal mice (Novitch et al., 1996; Schneider et al., 1994).

As a model system, cells of the human osteosarcoma cell line SAOS-2 are especially useful for the analysis of pRb's pleiotropic functions since they lack full-length nuclear pRb and produce instead a truncated 90 kd protein that is localized in the cytoplasm (Shew et al., 1990). Furthermore, in SAOS-2 cells the G1 growth arrest caused by pRb expression is accompanied by a distinctive morphological change that appears several days after introduction of wild-type pRb (Huang et al., 1988; Templeton et al., 1991; Hinds et al., 1992). This phenotype is characterized by spreading, a cessation of growth, and a senescent-appearing morphology (Templeton et al., 1991; Hinds et al., 1992; Qin et al., 1992). This morphological alteration is not exhibited upon introduction of mutant RB alleles derived from human tumors (Templeton et al., 1991; Mittnacht et al., 1991; Hinds et al., 1992), nor can it be observed with dominant-negative E2F proteins or p107 overexpression, despite the probable mechanistic similarities in their ability to block progression into S phase (Zhu et al., 1993; Qin et al., 1995). Therefore, pRb may influence the expression of genes controlling cell morphology in a manner distinct from simple repression of E2F-dependent promoters.

In attempt to explore the biochemical basis of pRb's functions and to produce a system to identify potential gene targets of pRb, we have generated a temperature-sensitive (ts) pRb. Characterization of this mutant supports a multifunctional role of pRb in suppression of cell proliferation, identifying both reversible and irreversible elements of growth arrest engendered by pRb. We suggest that pRb expression in this system not only prevents E2F function, but produces (possibly E2F-independent) cellular changes akin to differentiation or senescence that cannot be overcome by simple loss of pRb function, leading instead to apoptosis when the cells are once again rendered pRb-null through temperature shift. Transient restoration and removal of the differentiation and

proliferation controls enacted by some tumor suppressor proteins may thus be a more potent method to eliminate tumor cells than that afforded by constitutive expression.

Results

Identification of a pRb protein temperature-sensitive for the induction of growth arrest.

Regulated ectopic expression of genes involved in proliferation control is useful for studying their immediate effects upon synthesis and loss. For example, both inducible and temperature-sensitive p53 genes have been useful in identifying functional and transcriptional targets of this important tumor suppressor (Michalovitz et al., 1990; Yonish-Ronach et al., 1991; Barak et al., 1993; El-Deiry et al., 1993; Okamoto and Beach, 1994; Wu and Levine, 1994; Chen et al., 1996). Because pRb's long half-life makes it an unattractive candidate for inducible systems, we attempted to identify a temperature-sensitive version of the protein that could be more rapidly regulated. Therefore, a series of in-vitro-mutagenized RB cDNAs was produced and tested for growth suppression in SAOS-2 cells at 37°C and 32.5°C (Mittnacht et al., 1991; Hinds et al., 1992; see Figure 1). To test the mutant proteins for their growth suppressing activity, we employed a flat cell assay that has been used previously for pRb (Hinds et al., 1992). Plasmids encoding the mutants shown in Figure 1, wild-type pRb or the naturally occuring mutant Δ22 (Horowitz et al., 1990) were individually introduced along with a puromycin resistance marker into SAOS-2 cells by calcium-phosphate-mediated transfection. Transfected cells were then cultured at 32.5°C or 37°C. Suppression of SAOS-2 cell proliferation was assessed by the appearance of flat cells 7-10 days after transfection. As shown in Figure 1, the four linker-insertion mutants were found to be inactive in growth suppression at 37°C, but one mutant was found to induce growth suppression at 32.5°C but not at 37°C. This mutant, XX668, carries a 12bp Xho I linker inserted in-frame into a Xmn I site in between codons 668 and 669

(see Figure 1). This results in the insertion of four new amino acids, RSSG, changing the sequence of this mutant at codon 668 to -er[RSSG]ll- (see Figure 1).

Quantitation of flat cell induction, shown in Figure 2A, revealed that XX668 at 37°C produces on average 20% of the number of flat cells than does wild-type pRb, but is essentially equivalent to wild-type pRb in flat cell production at 32.5°C. As a further measure of the ability of XX668 to suppress proliferation, colony formation assays were performed. Figure 2B demonstrates that XX668 suppressed outgrowth of SAOS-2 cell colonies with wild-type efficiency at 32.5°C, but was unable to do so at 37°C. To demonstrate conclusively that XX668 behaves as a temperature-sensitive (ts) allele not only in the production of flat cells but also in the induction of G1 arrest in SAOS-2 cells, we used a modified flow cytometry technique that was reported previously for pRb and p107 (Zhu et al., 1993). XX668 or control vectors were cotransfected into SAOS-2 cells with a plasmid expressing the cell-surface marker CD20. Two days after transfection, cells that contained transfected DNA were identified by staining with an anti-CD20 monoclonal antibody, and the DNA content of the transfected cells was determined by propidium iodide staining. As shown in Figure 2C, transfection of XX668 was unable to efficiently alter the G1 fraction of SAOS-2 cells at 37°C, whereas even low amounts of the wild-type pRb cDNA lead to a significant increase in the G1 population. A slight increase in the G1 population was seen when XX668 was transfected in a very high concentration, consistent with its partial ability to induce flat cell formation. In contrast, at 32.5°C, XX668 and wild-type pRb both very efficiently blocked SAOS-2 cells in the G1 phase of the cell cycle. Thus XX668 is defective in flat cell formation, colony reduction and induction of G1 arrest when expressed at 37°C, but is indistinguishable from wild-type pRb at 32.5°C. In contrast, the tumor derived mutant Δ22 was found to be nonfunctional in these assays at either temperature (see

Figures 2 A, B and C), consistent with previous reports classifying Δ22 as a "loss of function" mutant (Horowitz et al., 1990; Templeton et al., 1991).

To exclude the possibility that the observed differences of XX668 in growth suppression and flat cell formation at 32.5°C and 37°C may be attributable to differences in transfection efficiencies or expression levels rather than to a functional temperature-sensitivity, we performed indirect immunofluorescence and immunoblotting. In a typical experiment, regardless of the temperature or whether XX668 or wild-type pRb-cDNA were used, approximately 30% of the transfected SAOS-2 cells became pRb-positive (data not shown). In addition, protein levels expressed from increasing amounts of XX668 or wild-type pRb-cDNA were essentially equal at both temperatures (Figure 3).

These data suggest that XX668 encodes a mutant pRb protein that is essentially wild-type for suppression of proliferation and flat cell formation at 32.5°C, but is only very weakly functional at 37°C, despite its persistence in the cell. XX668 therefore can be considered a temperature-sensitive allele of RB1.

Biochemical properties of tspRb.

We explored the biochemical properties of tspRb in effort to understand the defect that inactivates the protein at the nonpermissive temperature. Using an in vitro binding assay, we first determined if tspRb's ability to associate with the pocket binding viral oncoprotein E1A could correlate with its temperature dependent growth suppressing function. Extracts from SAOS-2 cells transfected with either XX668, wild-type pRb or Δ22 and cultured at 37°C or 32.5°C, were mixed with E1A that was made as a GST

fusion protein in Escherichia coli. The fraction of pRb that was bound by the GST-E1A protein was visualized by immunoblot with a pRb-specific monoclonal antibody (Figure 4). Equal amounts of input pRb protein were assured by performing direct immunoblots with aliquots of the cell extracts used in the in vitro binding experiments (Figure 4, Input). Regardless of the temperature, only approximately 10% of tspRb was associated with the E1A oncoprotein when compared to wild-type pRb. We conclude that the four amino acid insertion in the B-pocket of tspRb severely compromises the ability of the mutant protein to associate with E1A at both the permissive and the nonpermissive temperatures. Further, based on the employed assay, there is no correlation between tspRb's ability to suppress cell growth and its ability to bind to the E1A oncoprotein.

In contrast to E1A binding, nuclear association of pRb is well-correlated to its functional state (Mittnacht and Weinberg, 1991; Mittnacht et al., 1991; Templeton et al., 1991). Functionally active pRb stably associates with nuclear structures, whereas pRb that has been inactivated by hyperphosphorylation or mutation fails to do so (Mittnacht and Weinberg, 1991). This biochemical property of pRb can be measured by treatment of the transfected cells with a detergent-containing buffer of low salt concentration. Under these conditions, functional pRb is retained in the nucleus, but inactive pRb is extracted (Mittnacht and Weinberg, 1991; Mittnacht et al., 1994). To examine this property of tspRb, SAOS-2 cells were transfected with XX668, wild-type pRb or control vectors, cultured for 2 days at 32.5 °C or 37°C and finally lysed in a hypotonic buffer containing 0.1% Triton X-100. Lysates then were fractionated by low speed centrifugation into a low salt soluble supernatant and an insoluble nuclear pellet fraction. Distribution of the pRb proteins to the fractions was determined by immunoblotting and is shown in Figure 5. As expected, wild-type pRb was predominantly found in the nuclear pellet fraction at

both temperatures, whereas the majority of the tumor derived mutant Δ22 was extracted into the soluble supernatant (low-salt extract). In contrast, tspRb was stably associated with the nucleus and therefore mainly detectable in the nuclear pellet fraction at 32.5 °C but not at 37°C, where it was efficiently extracted into the low-salt fraction. Thus, tspRb encoded by XX668 is temperature-sensitive for nuclear tethering, and consistent with previous studies of tumor-derived mutants (Mittnacht and Weinberg, 1991; Mittnacht et al., 1991; Kratzke et al., 1994), this activity correlates well with its growth suppressing function.

Viral oncoprotein binding to pRb is thought to mimic and to compete with the association of cellular proteins that can bind to the pocket domain of pRb. The best understood cellular targets of pRb are the members of the E2F family of transcription factors. Since physical association between E2F and pRb is postulated to result in transcriptional repression and growth arrest, we wished to determine if the defects in E1A binding, growth suppression and nuclear tethering displayed by tspRb were associated with an inability to suppress transcription by E2F. To determine the functional consequence of tspRb expression on E2F dependent transcription, we employed a reporter consisting of the luciferase gene driven by the E2F-1 promoter. The E2F-1 promoter fragment used in this experiment has been shown to be subject to repression by pRb (Sellers et al., 1995). This reporter construct was cotransfected with tspRb cDNA, wild-type pRb, or control plasmids into SAOS-2 cells and the cells were incubated at either 37°C or 32.5°C. As shown in Figure 6, increasing amounts of tspRb were much less effective in repressing the E2F-1 promoter at 37°C, but gained this ability with wild-type efficiency at 32.5°C. This result clearly demonstrates a temperature dependent activity of tspRb in suppression of E2F-dependent transcription.

Inactivation of tspRb leads to DNA synthesis and apoptotic cell death.

The data obtained so far clearly demonstrate that tspRb in SAOS-2 cells can induce cell cycle arrest, a senescence-like morphology and E2F repression in a temperature-dependent manner. We next wished to determine if tspRb produced at the permissive temperature could be inactivated by a shift to the nonpermissive temperature, and ultimately wished to study the reversibility of the pRb phenotype. To determine if active tspRb can be inactivated after transient transfection of SAOS-2 cells, tspRb-induced, growth arrested flat cells produced by a one-week incubation at the permissive temperature were shifted back to the nonpermissive temperature and scored for re-entry into S-phase by measuring the ability to incorporate bromodeoxyuridine (BrdU) (Figure 7). Flat cells produced by wild-type pRb were found to be incapable of incorporating BrdU at either temperature, consistent with their arrest in G1 (or G0). Similarly, tspRb-produced flat cells at 32.5°C could not incorporate this nucleotide. Strikingly, when the latter were shifted to 37°C, approximately 30% of the cells were found to incorporate detectable amounts of BrdU as early as 24 hours after the shift. In contrast, growth-arrested cells produced by wild-type pRb expression at 32.5°C did not regain an ability to incorporate BrdU upon temperature upshift. Parental SAOS-2 cells permanently cultivated at 32.5°C and 37°C and XX668-transfected SAOS-2 cells permanently cultivated at the nonpermissive temperature were used as a positive control for BrdU incorporation (Figure 7). Thus, the block to S-phase entry caused by tspRb appears to be rapidly reversible by temperature shift.

The apparent reversibiliy of the S-phase block induced by tspRb allowed us to ask if the transiently growth-arrested SAOS-2 cells could permanently re-enter the cell cycle

and/or reverse the senescence-like morphology. Incorporation of BrdU into the tspRb transfected, temperature-shifted SAOS-2 cells occured while the cells still possessed the flat cell morphology. To determine if inactivation of tspRb would allow the cells to regain their normal shape and proliferation capacity, flat cells were produced by transient transfection of SAOS-2 cells with XX668 and wild-type pRb followed by drug selection for 7 to 10 days at 32.5°C. Flat cells then were removed from the drug and either further incubated at 32.5°C or shifted to 37°C. Photographs of cells within a fixed area of 0.25 cm2 were taken and the cells were counted on 7 consecutive days (see Table 1). While the number of wild-type transfected cells at both temperatures as well as tspRb-expressing cells at 32.5°C remained unchanged, tspRb-expressing flat cells at 37°C quickly disappeared from the plate, with loss of approximately fifty percent of the cells occuring within 24 hours and loss of all cells occurring within 7 days after temperature shift. Periodic visual inspection of the remaining flat cells suggested that nuclear division commonly precedes the death of these cells, perhaps consistent with their ability to re-enter S-phase prior to death (data not shown). Thus, flat cells produced by transient transfection with tspRb at the permissive temperature appear to display a reversible block to S phase entry, but are terminally blocked from proliferation, since they die after shift to the nonpermissive temperature.

We thought it possible that this death might result from a restoration of some pRb-regulated proliferation signals in the continued presence of anti-proliferative signals perhaps related to the cell shape. If so, such an "imbalance" might lead to apoptotic cell death. To provide evidence that the observed cell death is apoptotic in nature, we first attempted to block it by introducing Bcl-2 or adenovirus E1B 19K protein along with pRb. Interestingly, cotransfection of these anti-apoptotic genes along with tspRb

prevented cell death upon temperature upshift (see Table 1) suggesting that the observed cell death is apoptotic.

To confirm that the observed death of the temperature-shifted tspRb expressing cells is apoptotic and to quantitate the percentage of apoptotic cells within the total flat cell population, we determined the DNA content of the affected cells by flow cytometry analysis following propidium iodide staining (Figure 8). The percentage of apoptotic cells in the wtpRb induced flat cell population was very low, usually less than 10%, regardless of the incubation temperature. Similary, flat cells produced with tspRb at 32.5°C did not contain very many apoptotic cells. In contrast, when the latter were shifted to 37°C a significant number of cells with sub-G1 DNA contents were observed 24 hours after the shift, representing some 40 % of the total cell population. At 48 hours post-shift the percentage of apoptotic cells was a little lower but still significant at 30 to 40% of the total cell population. Co-introduction of either Bcl-2 or E1B 19K lead to a three to five-fold decrease in apoptotic cells. Furthermore, as a consequence of the additional expression of either apoptosis inhibitor, small, proliferating SAOS-2 cells, positive for the expression of tspRb were generated (Figure 9).

Thus, cellular changes caused by prolonged pRb expression appear to preclude proliferation (but not cell cycle progression) upon removal of pRb as a result of programmed cell death. Inhibition of this death by known protein inhibitors of apoptosis allows the outgrowth of colonies of pRb-positive, morphologically "normal" SAOS-2 cells, suggesting that the flat cell phenotype can be reversed once the apoptotic signals are overcome.

Discussion

We have produced a genetically engineered pRb-mutant, XX668, carrying an insertion of four amino acids at codon 668 in the B-pocket, that can exert very diverse effects on transiently transfected SAOS-2 cells, depending on the temperature at which the culture is maintained. At 37°C, despite its persistence in the cell, XX668, unlike wild-type pRb, is defective in flat cell formation, colony reduction and induction of G1 arrest. In this respect, it behaves like tumor mutants described here and in earlier work (Templeton et al., 1991; Hinds et al., 1992). However, unlike these tumor mutants, XX668 was found to be indistinguishable from wild-type pRb for suppression of proliferation when the cells were kept at 32.5°C. Furthermore, at this temperature it can very efficiently induce flat cell formation. Thus, XX668 encodes a mutant pRb protein that is essentially wild-type at the low temperature, but is only very weakly functional at 37°C. These findings strongly suggest that XX668 is a temperature-sensitive (ts) mutant of pRb.

Not surprisingly, the insertion in the B-pocket of tspRb severely compromised its ability to interact with E1A. tspRb interacts only weakly with E1A at 37°C, and this binding cannot be improved upon changing the temperature at which the protein is produced. Therefore, binding to E1A seems not to correlate with pRb's ability to suppress proliferation. This is consistent with previous studies in which pRb mutants with mutations within the B-pocket were reported that retained the ability to suppress cell proliferation but failed to associate with E1A (Mittnacht et al., 1991; Kratzke et al., 1994). The XX668 mutant is also severely compromised in its ability to associate with the nucleus at 37°C, a loss-of-function common to inactive, tumor-derived mutants (Mittnacht and Weinberg, 1991; Mittnacht et al., 1991; Templeton et al., 1991).

However, in contrast to E1A binding, tspRb produced at 32.5°C regains the ability to associate with the nucleus, suggesting a temperature-dependent structural alteration of the pocket region required for nuclear tethering.

It is believed that transcriptional repression of genes containing E2F sites mediated by complexes of pRb and E2F contributes significantly to pRb's role as a tumor suppressor. Consistent with this, we find that tspRb is much less effective than wild-type pRb in repressing the E2F-1 promoter at the nonpermissive temperature, but gains this ability with wild-type efficiency at 32.5°C. This result clearly suggests a temperature dependent activity of tspRb in suppression of E2F-dependent transcription, correlating with its nuclear tethering and growth suppressing activity. TspRb should therefore be a very useful tool for identifying genes that are turned on or off soon after temperature shift, and which would thus be good candidates for direct control by pRb. To this end, we have recently been able to establish cell lines stably expressing tspRb that undergo growth arrest at the permissive temperature (FT and PH, unpublished observations). Interestingly, our preliminary data suggests that the inability of tspRb to suppress transcription at the nonpermissive temperature is not simply due to a defect in association with E2F, since we have observed equivalent, low levels of pRb/E2F complexes at both the permissive and nonpermissive temperatures (FT and PH, unpublished observations). The XX668 mutation may be somewhat similar to a recently-identified, low penetrance allele of RB1, called 661W, that is defective for E1A and E2F association, yet retains the ability to arrest cells (Kratzke et al., 1994).

We suspect that the temperature dependence of tspRb's ability to repress E2F-dependent transcription may be due to a defect in its ability to interact with the

transcriptional machinery adjacent to the E2F binding sites. It has been proposed that the pRb-pocket, after being tethered to a specific promoter through E2F, could either bind surrounding transcription factors, preventing their interaction with the basal transcription machinery (TFIID like factors; the factor PU.1) (Chow and Dean, 1996), or bind one or more unknown proteins "X" that has an intrinsic repressor activity (Sellers et al., 1995). Both possibilities would result in the repression of transcription of the affected gene. tspRb may be unable to form such contacts at the nonpermissive temperature, but regain this ability at the permissive temperature. The suppression of proliferation mediated by tspRb at the permissive temperature may thus result from one or more pRb-containing multiprotein complexes that act to repress the transcription of growth-promoting genes. Clearly, our knowledge of pRb's interaction with E2F only scratches the surface of pRb's true molecular role in proliferation control. The tspRb protein promises to be a powerful tool to unravel the molecular mechanism of pRb-mediated transcriptional repression.

The utility of tspRb as a reagent to study pRb's molecular functions in tumor suppression is enhanced by its apparent ability to be efficiently inactivated by temperature upshift. Cell cycle re-entry of tspRb-arrested SAOS-2 cells occurred well within 24 hours as determined by BrdU incorporation, suggesting pRb's control of E2F and other factors is rather rapidly reversible. Most interestingly, although these cells displayed a reversible block to S phase entry, they were terminally blocked from proliferation, since they died by apoptosis after shift to the nonpermissive temperature. An intriguing property of pRb-expressing SAOS-2 cells is the appearance of the so-called flat cell phenotype (Templeton et al., 1991; Hinds et al., 1992; Zhu et al., 1993; Qin et al., 1995). It was suggested that these flat cells resemble the senescent phenotype of primary fibroblasts seen after extended time in culture (Templeton et al.,

1991), and that this biological activity of pRb may be a manifastation of a less reversible type of exit from the cell cycle leading to senescence or differentiation (Weinberg, 1995; Qin et al., 1995). Prolonged expression of pRb may produce a "terminal" pseudo-differentiated or senescent state characterized by the flat cell phenotype that is distinct from pRb/E2F-type growth suppression and which is poorly reversible upon loss of pRb. Indeed, some property of the flat cells themselves, such as cytoskeletal structure or cell shape, may directly contribute to the relative irreversibility of the arrested state. Thus, cellular changes caused by the prolonged expression of functional tspRb appear to preclude rather than restore proliferation (but not cell cycle progression) upon removal of a functional pRb as a result of programmed cell death. Interruption of this apoptotic response could be activated by cotransfection of Bcl-2 or E1B19K, which led to the outgrowth of small, proliferating SAOS-2 cells, positive for the expression of tspRb.

Given these results, it is of note that SAOS-2 cells express high levels of the CDK inhibitor gene encoding p16, which is overexpressed in senescent cells, and which is one of the candidate causal senescence genes (Smith and Pereira-Smith, 1996). Since loss of pRb in SAOS-2 cells may allow bypass of the putative p16 function in senescence (Sherr, 1996), reintroduction of a hypophosporylated form of pRb in pRb-negative tumor cells could promote a terminal cell cycle exit by switching on elements of a senescence program. The functional inactivation of pRb once the senescent phenotype is established may result in two conflicting signals: (i) a growth promoting signal triggered by a deregulated E2F and (ii) a growth inhibitory signal maintained by the senescence machinery. As a consequence of these conflicting signals the cells undergo apoptosis, since the inactivation of pRb in cells already committed to senescence may be similarly insufficient to promote proliferation. In support of this are

studies that suggest several proteins that play a role in regulating cell cycle progression also have an apoptotic potential and that the apoptotic signal induced by such proteins is a direct consequence of conflicting growth control functions. Of particular interest are recent studies that suggest a p53-independent apoptotic activity of E2F-1 that is dependent on the deregulation of a functional pRb-E2F-1 repressor complex. Thus, derepression of genes containing E2F sites may lead directly to apoptosis if the cell's interpretation of its environment is otherwise at odds with a decision to proliferate (Hsieh et al., 1997; Phillips et al., 1997).

In summary, we propose that the introduction of a functional pRb protein into SAOS-2 cells acts as a negative epigenetic signal that is followed by the start of a senescence program (or alternatively a differentiation program) that in turn causes the observed S phase block accompanied by the shape change of the cells. Once the growth inhibitory signal is established, removal of functional pRb restores S phase entry but this may conflict with irreversible negative signals resulting from other downstream effects of pRb with the consequent death of the affected cells. Achieved here with a temperature-sensitive pRb, such a transitory reactivation of the pRb pathway has broader conceptual implications for cancer therapy, since techniques designed to only temporarily restore the pRb pathway in tumor cells may prove more efficient at cell killing than permanent expression of a given cell cycle regulator.

Materials and methods

Cell Culture, Plasmids, and Transfections.

The human osteosarcoma cell line SAOS-2, subclone 2.4 (Hinds et al., 1992) was used for all studies. Cells were maintained in Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 15% heat-inactivated fetal bovine serum in a 3% CO_2 incubator at 37°C.

All pRb expression plasmids were constructed in pSVE (Templeton et al., 1991). The human pRb expression vector phRbc-SVE (here referred to as wtpRb) has been described previously (Templeton et al., 1991; Hinds et al., 1992) and was used in all transfection experiments to express wild-type pRb. Several mutant cDNAs were constructed in this vector by inserting a XhoI linker in frame into different restriction sites at certain codons (Mittnacht et al., 1991; Hinds et al., 1992). HX108 has a 12 bp linker inserted at the HincII site at codon 108, resulting in the insertion of four amino acids (P-L-E-R) at this position. AcX414 has an insertion of four amino acids (I-P-R-G) at codon 414, resulting from a 10 bp linker inserted into an AccI site. The mutant XX668 carries the 12 bp linker inserted at the XmnI restriction site at codon 668, with four new amino acids (R-S-S-G) at this position. AX763 was constructed by inserting a 12 bp linker into a AlwNI site at codon 763, and has three new amino acids (P-L-E) at this position.

The expression vector pSVΔ22 encoding a pRb protein deleted in exon 22 (here referred to as Δ22) was used as a negative control for pRb expression and function.

The Δ22 cDNA was derived from the NCI-H592 small cell lung carcinoma (Horwitz et al., 1990) and subcloned into pSVE (Templeton et al., 1991).

The vector pBabepuro (Morgenstern and Land, 1990) was used to mediate resistance to puromycin.

SAOS-2 cells (1-2x10^6) were transfected with the indicated plasmids on 10 cm dishes for 18hrs by using the 2x Bes-buffered saline (2xBBS)/calcium phosphate method of Chen and Okayama (1987), with modifications as described previously (Hinds et al., 1992).

Flat cell assay and colony formation assay

For flat cell assays and colony formation assays, cells were transiently transfected with 1.5µg of pBabepuro and 20µg of the indicated pRb expression plasmid. Following transfection, cells were plated at 5x10^5 per 10cm dish. Puromycin (Sigma) was added at 0.5µg/ml 24 hours after plating and cells were selected at either 37°C or 32.5°C. After 7-10 days of selection, cells were stained with crystal violet and flat cells were quantitated as described by Hinds et al. (1992). For the quantitation of colonies, cells were stained with crystal violet after 14 days of selection, when macroscopic colonies became detectable. Since no macroscopic colonies were detectable at this time on plates maintained at 32.5°C, microcolonies were quantitated as described for the flat cells.

Flow cytometry analysis

Flow cytometry analysis for the determination of cell cycle profiles of pRb transfected cells was performed as described previously for pRb and p107 by Zhu et al. (1993). The expression plasmid for the B cell surface marker CD20 (pCMVCD20) was used in cell cycle analysis (van den Heuvel and Harlow, 1993). Briefly, 2 µg of pCMVCD20 was cotransfected with the indicated amounts of pRb expression constructs into SAOS-2 cells. 48 hours after the removal of DNA precipitates, cells were rinsed off the plates with PBS containing 0.1% EDTA, pelleted, and stained with 20 µl of a FITC-conjugated anti-CD20 monoclonal antibody (PharMingen). Subsequently, cells were fixed with 90% ethanol on ice for several hours. Before flow cytometry analysis, the cells were treated with 200 µg/ml RNase A for 15 minutes at 37°C and stained with a solution containing 20 µg/ml of propidium iodide. Flow cytometry analysis was performed on a Becton-Dickinson FACScan. The intensity of propidium iodode staining was analyzed with the CellFIT Cell Cycle Analysis software to determine the DNA content and hence the cell-cycle profiles on cells that were positive for FITC staining. The data presented are representative of multiple experiments. We observed small variations in the G1, S, and G2/M populations between samples that were independently transfected with the same plasmids. However, the differences between controls and testing samples observed in each particular experiment were significantly consistent in all separate experiments.

Immunoblotting and in vitro binding assay

The expression of transfected cDNAs was monitored by immunoblotting. Aliquots of SAOS-2 cells, transfected with the indicated amounts of pRb expression constructs for

cell cycle analysis, reporter assays or in vitro binding assays, were lysed in ELB (50 mM HEPES, pH 7.2; 250 mM NaCl; 2 mM EDTA; 0.1 % Nonidet P-40) 48 hours after the removal of DNA precipitates as described (Latham et al., 1996). Protein concentrations in the cell lysates were determined by the Bio-Rad protein assay. Proteins were separated by SDS-PAGE and transferred to nitrocellulose by standard procedures. pRb proteins were monitored by blotting with the monoclonal antibodies 245 (PharMingen) or Ab-5 (Oncogene Science). Detection was performed, after incubation with peroxidase-conjugated donkey anti-mouse IgG (Jackson Immunoresearch), by enhanced chemiluminescence (Amersham), as described previously (Latham et al., 1996).

Plasmids encoding the glutathione S-transferase (GST) $E1A_{12S}$ and $E1A_{12S}$ pm928/961 fusion proteins (Kraus et al., 1994) were used to produce the proteins for the GST-pull down experiments. Glutathione S-transferase fusion proteins were induced, purified and recovered on glutathione-Sepharose beads as described earlier (Kaelin et al., 1991). The beads were rocked with aliquots of cell lysates for 2 hours at 4°C and then washed five times with ELB. The beads were then boiled in sample buffer and bound proteins were resolved by SDS-PAGE. pRb proteins were visualized by immunoblotting as described above. With each aliquot of cell lysate approximately 150 µg of total protein was analyzed.

Nuclear Extraction

SAOS-2 cells were transfected with 20 µg of the indicated pRb expression construct and incubated at either 37°C or 32.5°C. 48 hours after the removal of DNA precipitates,

transfected cells were subjected to subcellular fractionation as described previously (Mittnacht and Weinberg, 1991). Briefly, cells were lysed in a hypotonic buffer (10 mM HEPES-KOH, pH 7.9; 10 mM KCl; 1.5 mM $MgCl_2$; 0.5 mM dithiothreitol) containing 0.1% Triton X-100, and fractionated by low speed centrifugation into a low salt soluble supernatant and an insoluble nuclear pellet. The insoluble nuclear pellet fraction then was further extracted with ELB. pRb in aliquots of both extracts was detected by immunoblotting as described above. To control for pRb expression, a small aliquot of each sample was directly lysed in ELB and monitored for pRb expression by immunoblotting.

E2F transcription assay

Luciferase assays using the luciferase reporter plasmid pGL2-AN, containing the E2F-1 promoter upstream of the luciferase cDNA (Neumann et al., 1994) were performed according to standard protocols (Ausubel et al., 1990). Cells were co-transfected with 5µg pGL2-AN, 2µg pCMV-ßgal and the indicated amount of different pRb expression plasmids and incubated at 37°C or 32.5°C. 48 hours after the removal of DNA precipitates, ß-galactosidase and luciferase activities were assayed. Luciferase values were normalized for ß-galactosidase activity and expressed relative to the activity observed for the reporter in the presence of pSVE only.

Analyses of DNA synthesis and cell death and immunocytochemical staining for pRb

SAOS-2 cells were transfected with 1.5µg of pBabepuro, 20µg of the indicated pRb expression plasmid and 5µg of either the Bcl-2 expression vector SFFV-Bcl-2 (kindly provided by Dr. S. Korsmeyer) or the E1B 19K expression vector pcDNA3-19K (Han et al., 1996), when indicated. Following transfection, cells were plated and selected at 32.5°C as described for the flat cell assays. After 7 to 10 days, when only flat cells appeared on the plates, cells were removed from the drug and either further incubated at 32.5°C or shifted to 37°C. Treated cells as well as control cell populations were then scored for (i) DNA synthesis by measuring their ability to incorporate bromodeoxyuridine (BrdU), for (ii) cell death by cell number comparison and flow cytometry analysis of propidium iodide-labeled cells and for (iii) pRb expression by immunocytochemical staining for pRb.

(i) BrdU incorporation was detected exactly as described earlier (Latham et al., 1996). At least 100 nuclei per sample were counted.

(ii) To compare cell numbers, photographs of cells within a fixed area of 0.25 cm2 were taken on 7 consecutive days and the cells in this fixed area were counted. Relative cell numbers based on several experiments are presented. Flow cytometry analysis was carried out and total flat cell populations were gated and analyzed as described (Phillips et al., 1997).

(iii) The immunocytochemical staining for pRb was performed as described (Latham et al., 1996).

Acknowledgments

We thank Drs. W.G Kaelin Jr., S. Korsmeyer, J.R. Nevins, S. van den Heuvel and E. White for generous gifts of plasmid reagents. This work was supported by grants RPG-95-013-03-CB from the American Cancer Society and DAMD17-94-J-4258 from the U.S. Army. P.W.H. is a Scholar of the Leukemia Society of America. F.T. was supported by his wife Dr. Beate Mueller-Tiemann and by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft.

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Table 1. Inactivation of pRb after flatcell induction results in cell death.

Flatcell phenotype induced at 32.5°C with [a]	Number of cells in % after further incubation at 32.5°C [b]		Number of cells in % after temperature shift to 37°C [b]			
	day 0	day 7	day 0	day 1	day 2	day 7
wtpRb	100	100	100	100	100	100
tspRb	100	100	100	55	45	0-1
tspRb+Bcl-2	100	100	100	100	100	150
tspRb+E1B 19K	100	100	100	100	100	140

Legend

a SAOS-2 cells were cotransfected with 1.5µg pBabepuro and 20µg of wtpRb or tspRb (XX668) and 5µg of Bcl-2 or E1B 19K, where indicated. Following transfection, cells were subjected to drug selection at 32.5°C. After 7 to 10 days, when only flat cells appeared on the plate, cells were removed from the drug and either further incubated at 32.5°C or shifted from 32.5°C to 37°C for up to 7 days.

b To compare cell numbers, photographs of cells within a fixed area of 0.25 cm2 were taken on 7 consecutive days and the cells in this fixed area were counted. Average cell numbers based on several experiments were calculated and are presented relative to the cell numbers on day 0 of either temperature which were set as 100%.

Figure legends

Figure 1. Structure of in-vitro-mutagenized pRb proteins and their ability for growth suppression at 37°C and 32.5°C. In-vitro mutants were constructed by oligonucleotide linker-insertion into restriction sites at certain codons of the human RB1 cDNA as described in Materials and methods. All mutants have been tested to express mutated forms of the pRb protein. The schematic diagram shows the human pRb protein and the positions where the XhoI linker was inserted in frame to generate the mutants HX108, AcX414, XX668 and AX763. The solid black boxes A and B represent the two regions of the retinoblastoma protein essential for binding to E1A and SV40 large T antigen (Hu et al., 1990), together known as the pocket domain of pRb. The shaded grey box represents the nuclear localization signal (NLS) of pRb (amino acids 860-876). To determine the ability of the generated in-vitro mutants to suppress growth at 37°C or 32.5°C, SAOS-2 cells were transfected with pBabepuro and HX108, AcX414, XX668, AX763, wtpRb or Δ22 and transfected cells were selected at 37°C or 32.5°C. Suppression of SAOS-2 cell growth was assessed by the appearance of large, apparently nondividing cells 7-10 days after transfection. (+) Large number of such cells. (-) No or very few enlarged cells were observed.

Figure 2. Temperature-dependent growth suppression by the pRb mutant XX668. For flat cell assays (A) and colony formation assays (B), cells were transiently transfected with 1.5μg of pBabepuro and 20μg of the indicated pRb expression plasmid. Following transfection, cells were plated at $5x10^5$ per 10cm dish, subjected to drug selection at either 37°C or 32.5°C and stained 7-10 days (A) or 14 days (B) later. Flat cells (A) or colonies (B) were counted as described in Material and methods. The graphs (A and

B) represent the mean of at least three independent experiments. For the flat cell assays (A), the percentage of flat cells relative to wtpRb (100%) is given in the graph. For cell cycle analysis (C) cells were cotransfected with 2 µg of pCMVCD20 and 20µg of pSVE or Δ22 or 5, 10 and 20µg of wtpRb or XX668. The total amount of plasmid DNA was kept constant in all samples (20µg) by adding pSVE plasmid DNA, where needed. Cells were incubated at 37°C or 32.5°C and harvested 48 hours post-transfection, stained for CD20 and DNA content, and analzyed by flow cytometry. The percentage of CD20-positive cells in G1 was plotted for each sample. The data presented are representative of multiple experiments.

Figure 3. Expression of wild-type and mutant pRb cDNAs in SAOS-2 cells. SAOS-2 cells, transfected as described in figure 2B with the indicated amounts of Δ22, wtpRb or XX668 and incubated for 48 hours post-transfection at 37°C or 32.5°C were lysed and aliquots were immunoblotted with an anti-pRb monoclonal antibody. The position of wtpRb and XX668 proteins (pRb) and the position of the slightly faster migrating Δ22 protein is indicated.

Figure 4. In-vitro binding of pRb proteins to E1A. SAOS-2 cells were transiently transfected with 20µg of Δ22, wtpRb or tspRb (XX668) and maintained for 48 hours post-transfection at 37°C or 32.5°C. Aliquots of the different cell lysates were incubated with glutathione-Sepharose loaded with GST-E1A_{12S} or GST-pm928/961 for 2 hours at 4°C. Bound proteins were separated by SDS-PAGE and immunoblotted for pRb. To control the input of the pRb proteins, aliquots of the same lysates were directly immunoblotted for pRb (input).

Figure 5. Nuclear Association of pRb. SAOS-2 cells were transfected with 20 μg of the indicated pRb expression construct and incubated at either 37°C or 32.5°C. 48 hours after the removal of DNA precipitates, transfected cells were subjected to subcellular fractionation. Aliquots of the low-salt extract fraction and the nuclear pellet fraction were analyzed for pRb by immunoblotting with an anti-pRb specific monoclonal antibody. The position of wtpRb and XX668 proteins (pRb) and the position of the slightly faster migrating Δ22 protein is indicated.

Figure 6. Repression of E2F-dependent transcription. SAOS-2 cells were co-transfected with 5μg pGL2-AN, 2μg pCMV-ßgal and 20μg of pSVE or Δ22 or 5, 10 and 20μg of wtpRb or XX668. The total amount of plasmid DNA was kept constant in all samples (20μg) by adding pSVE plasmid DNA, where needed. Following transfection cells were incubated for 48 hours at 37°C or 32.5°C, cell extracts were prepared and ß-galactosidase and luciferase activities were assayed. Luciferase values were normalized for ß-galactosidase activity and plotted relative to the activity observed for the reporter in the presence of pSVE. Data shown are representative of three experiments.

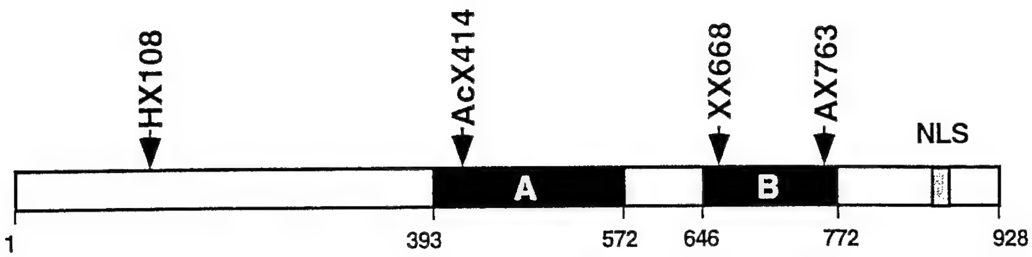
Figure 7. Re-entry of tspRb induced growth arrested flat cells into S phase upon temperature shift to the nonpermissive temperature. SAOS-2 cells were cotransfected with 1.5μg pBabepuro and 20μg of wtpRb or tspRb (XX668). Following transfection, cells were subjected to drug selection at 32.5°C. In addition, tspRb transfected cells were selected at 37°C. After 7 to 10 days, when only flat cells appeared on the plates,

cells were removed from the drug and either further incubated at their former temperature or shifted from 32.5°C to 37°C for 24 or 48 hours. Shifted and indicated control cells were scored for re-entry into S phase by measuring their ability to incorporate bromodeoxyuridine (BrdU). The percentage of BrdU-incorporating cells for each sample is shown in the graph. The results shown represent three independent experiments and the error bars indicate the standard deviations. Representive pictures of all samples stained for BrdU were taken at the same magnification.

Figure 8. Inactivation of tspRb after induction of the flat cell phenotype leads to apoptosis. SAOS-2 cells were cotransfected with 1.5μg pBabepuro and 20μg of wtpRb or tspRb (XX668) and 5μg of Bcl-2 or E1B 19K, where indicated. Following transfection, cells were subjected to drug selection at 32.5°C. After 7 to 10 days, when only flat cells appeared on the plate, cells were removed from the drug and either further incubated at 32.5°C or shifted from 32.5°C to 37°C. WtpRb and tspRb transfected cells that were shifted from 32.5°C to 37°C were harvested for flow cytometry analysis 24 and 48 hours after temperature shift. All other cells were harvested for flow cytometry analysis 48 hours after the former were shifted. Total flat cell populations were analyzed. Apoptosis was measured by the accumulation of cells with a sub-G1 DNA content in an area indicated as M1. A representative FACS analysis is shown (A). The graph (B) represents the percentage of cells with sub-G1 content (apoptotic cells) with mean values derived from three independent experiments.

Figure 9. Inhibition of cell death by apoptosis inhibitors allows the outgrowth of pRb-positive SAOS-2 cells with a reversed cell morphology. SAOS-2 cells transfected with

tspRb and the apoptosis inhibitors Bcl-2 or E1B 19K, respectively, or with tspRb or wtpRb that were generated at day 7 in the experiment described in Table 1, were immunocytochemically stained for pRb and compared to SAOS-2 cells that were cultivated at 37°C or 32.5°C. Representive photographs were taken at the same magnification.



Protein	Mutation	Growth suppression at 37 °C	Growth suppression at 32.5 °C
HX108	RV**PLER**DL 108	-	-
AcX414	S**IPRG**ILK 414	-	-
XX668	ER**RSSG**LL 668	-	+
AX763	Q**PLE**RL 763	-	-
wtpRb	-	+	+
Δ22	deletion of exon 22	-	-

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Figure 1.

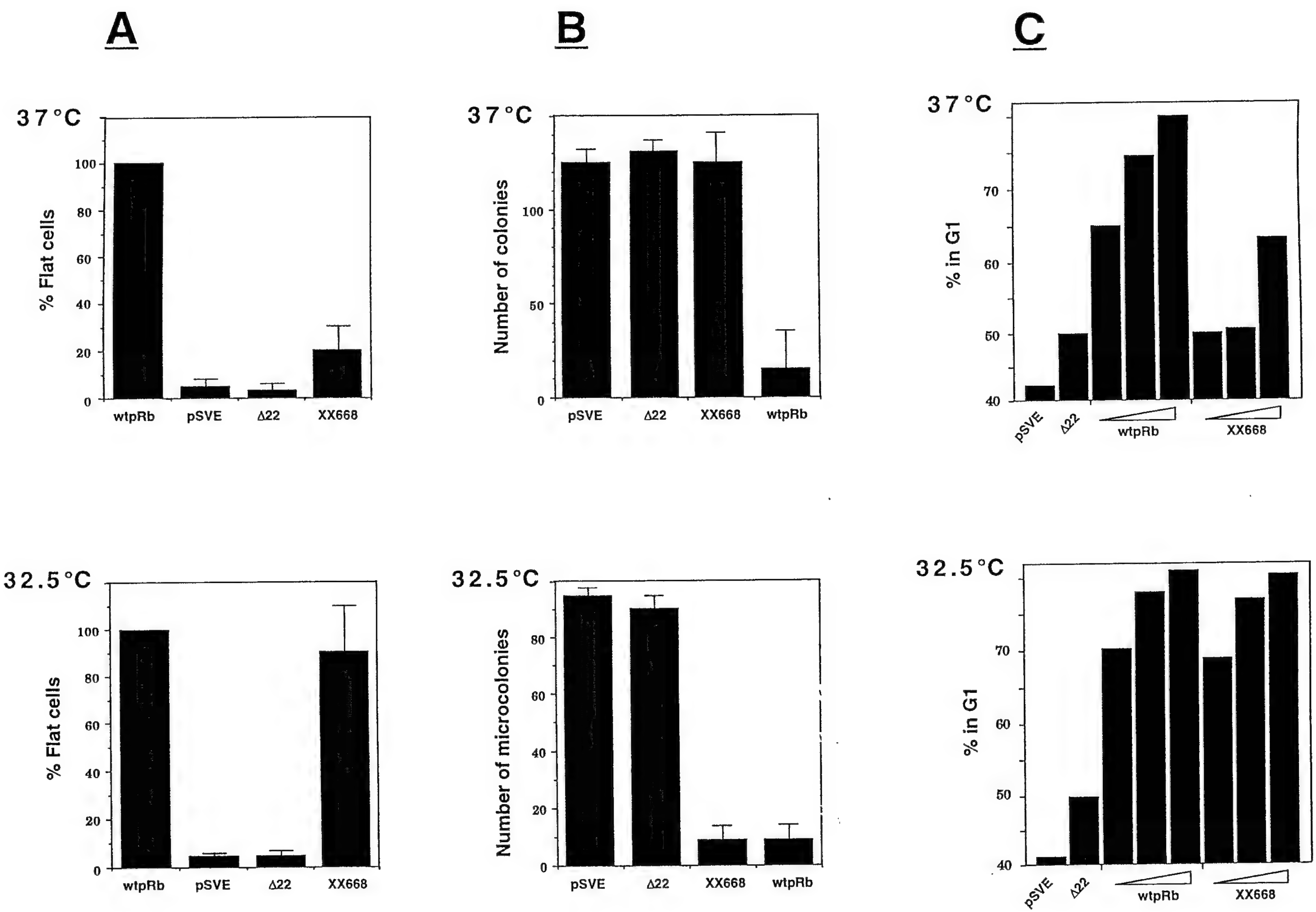

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Figure 2.

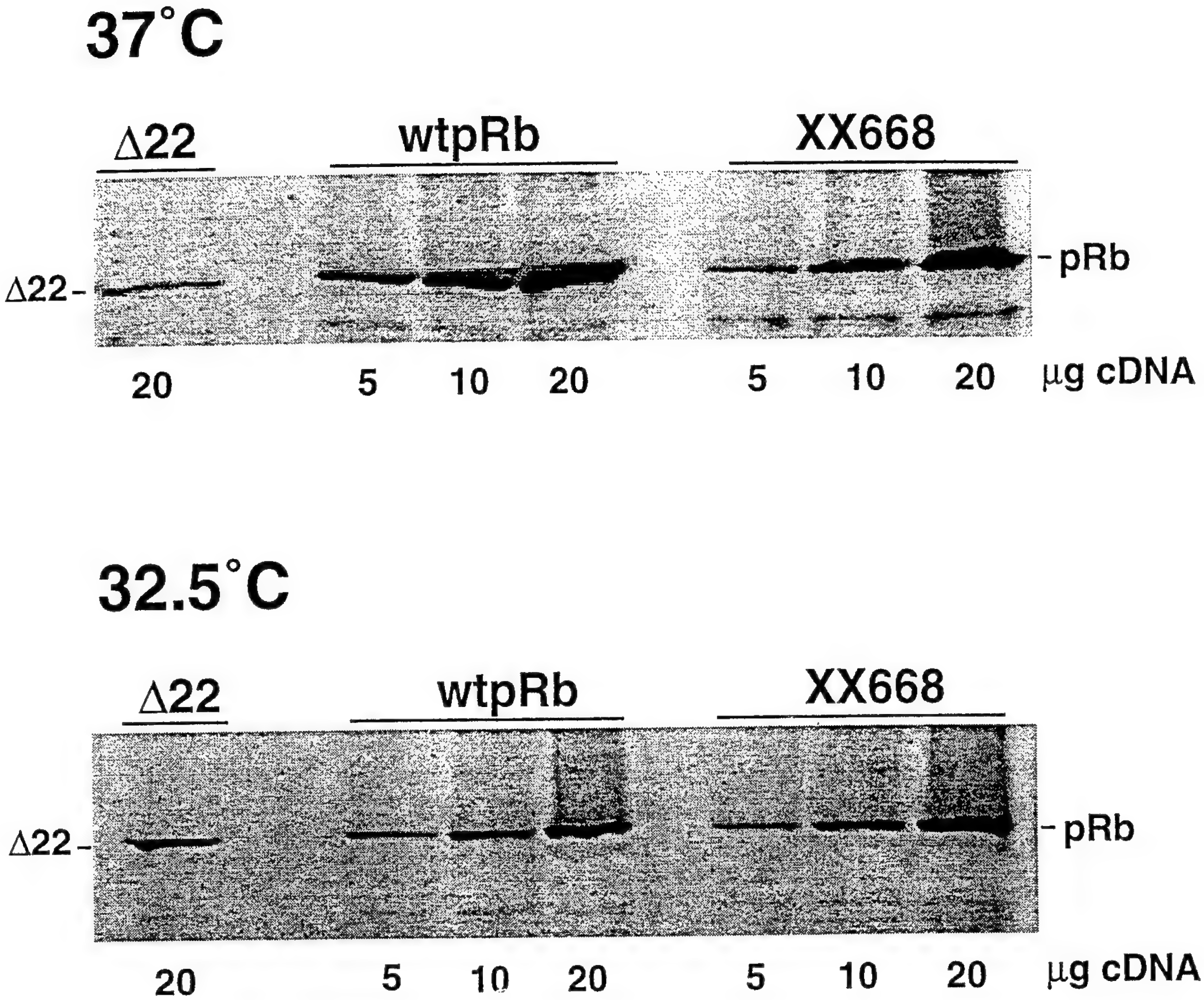

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Figure 3:

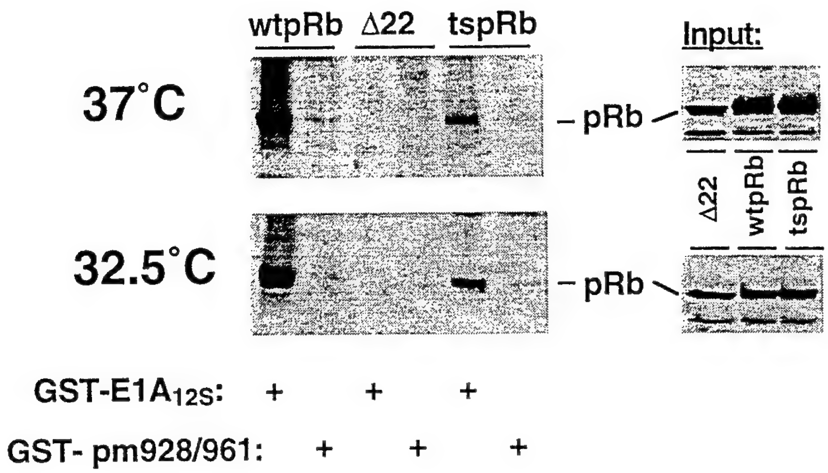

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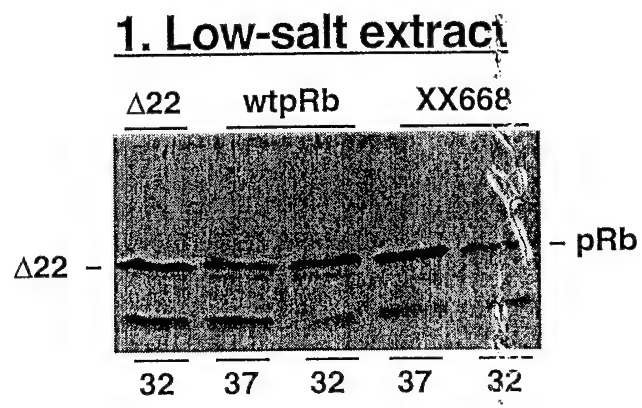
Figure 4.



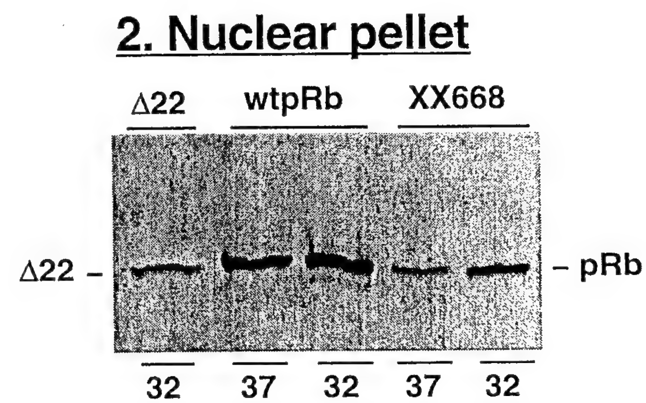


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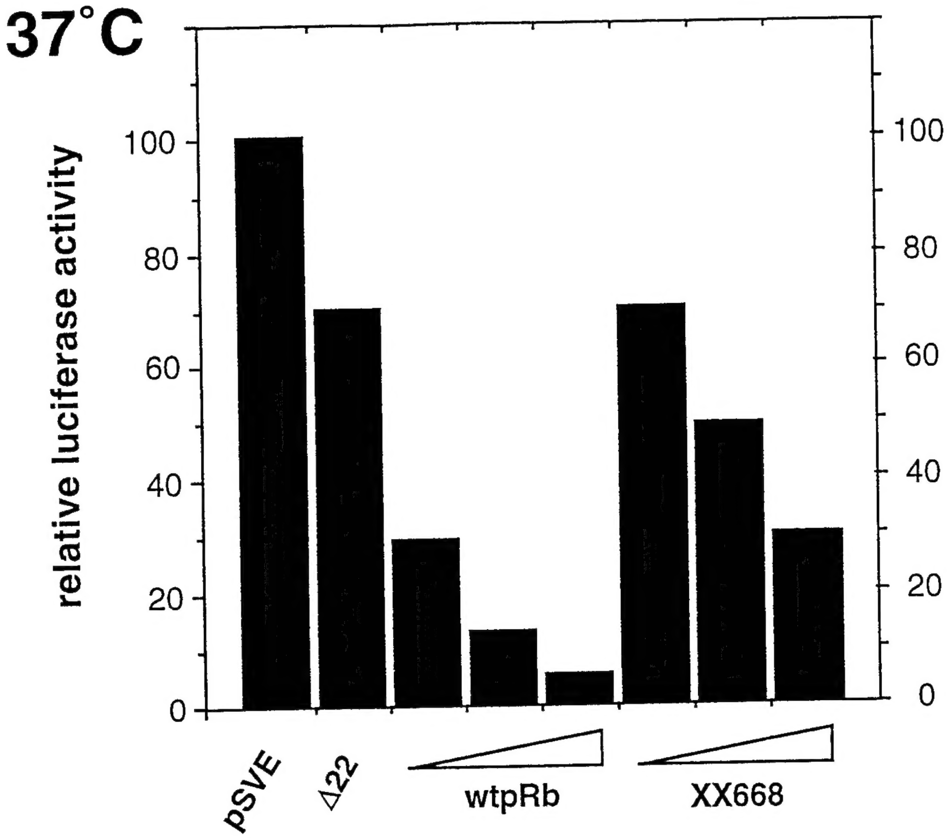
Figure 5.



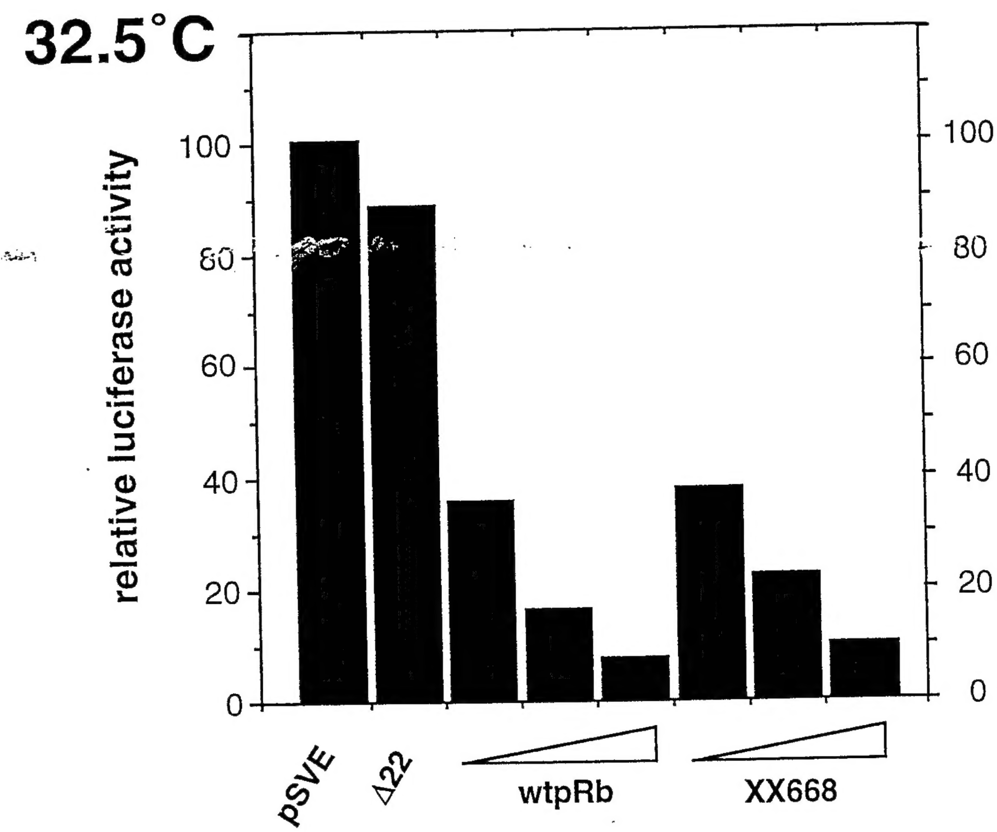


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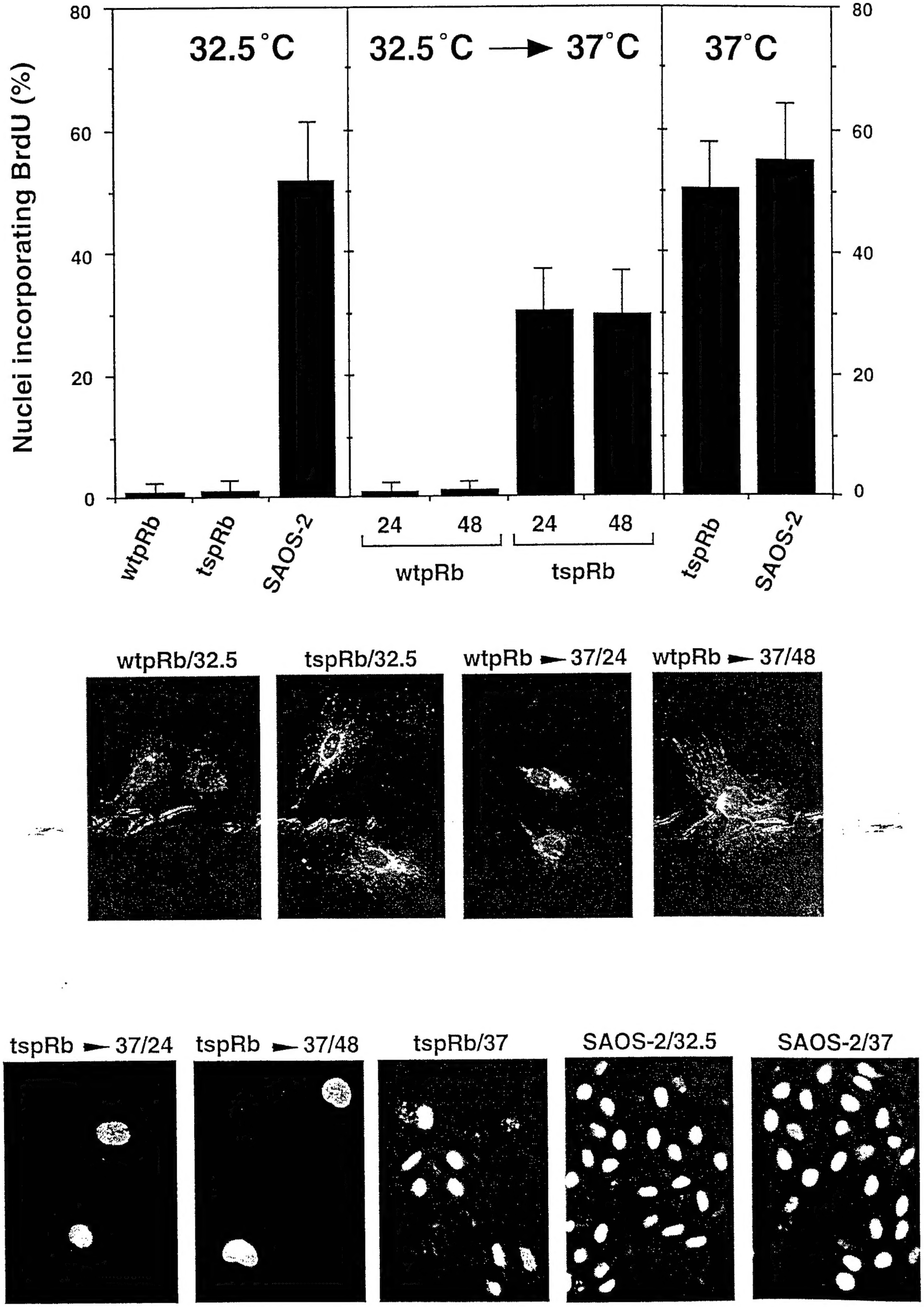
Figure 6.


Nuclei incorporating BrdU (%)
80
60
40
20
0
32.5°C
32.5°C ⟶ 37°C
37°C
wtpRb
tspRb
SAOS-2
24
48
wtpRb
24
48
tspRb
tspRb
SAOS-2
wtpRb/32.5
tspRb/32.5
wtpRb ► 37/24
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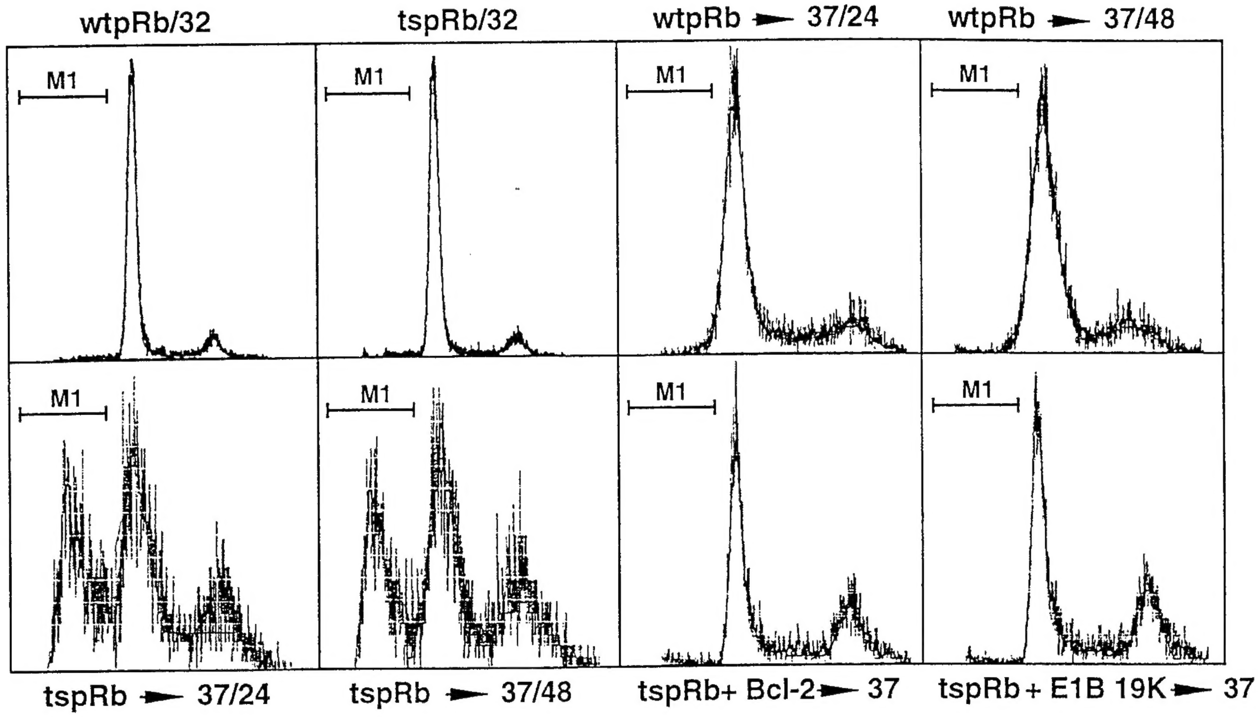
SAOS-2/37

A



B

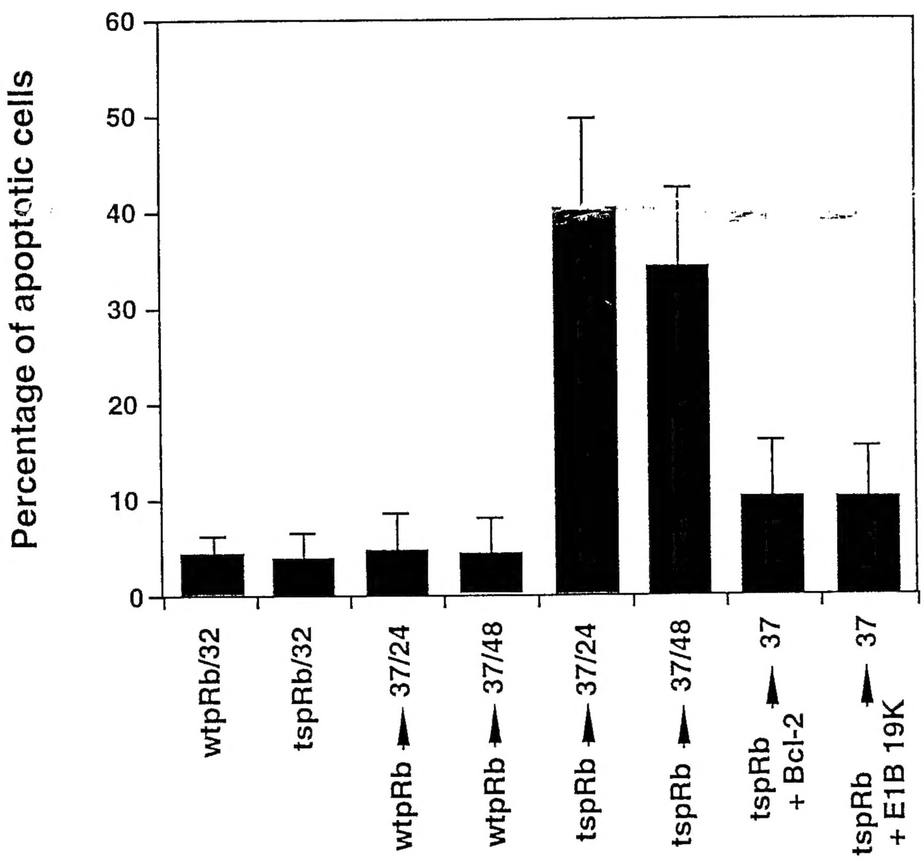


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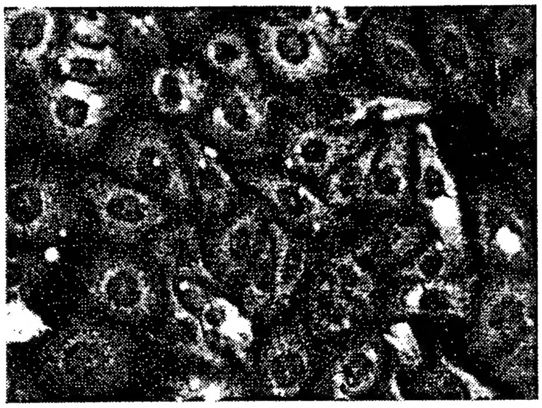
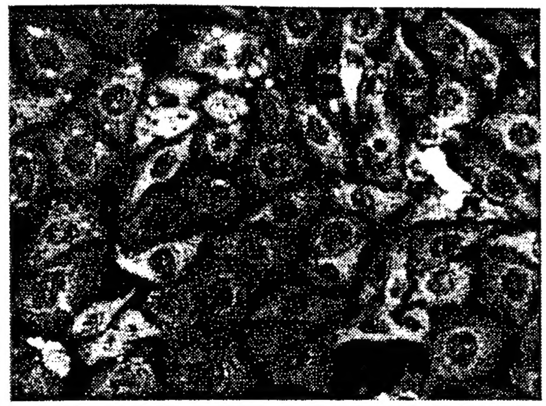
Figure 8.

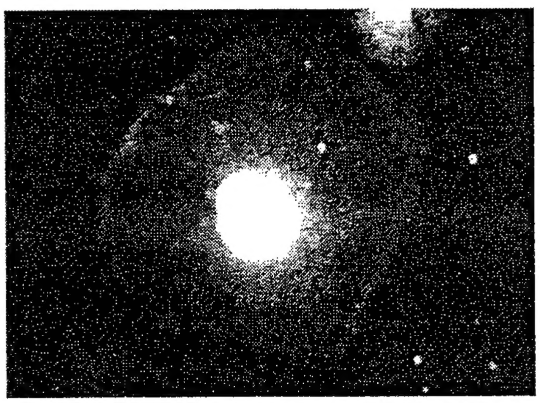
SAOS-2/32.5



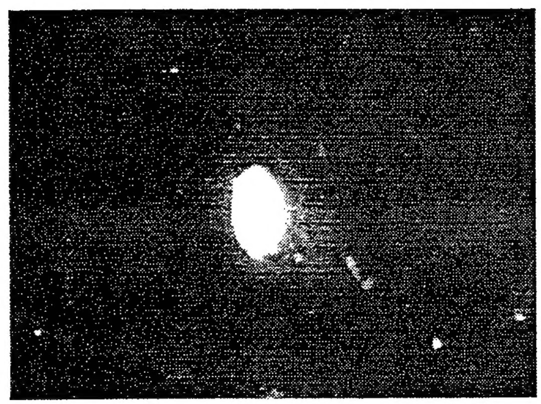
SAOS-2/37



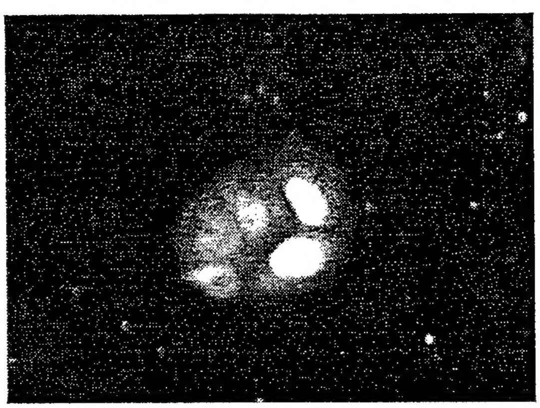
tspRb/32.5



wtpRb/32.5



tspRb + Bcl-2 → 37



tspRb + E1B 19K → 37

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Figure 9.